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Verapamil and diltiazem inhibit receptor-operated calcium channels and intracellular calcium oscillations in rat hepatocytes

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Fura-2 loaded rat hepatocytes were used to determine whether the L-type channel blockers, verapamil and diltiazem, affect receptor-operated calcium channels (ROCCs). The flux through ROCCs was followed by quenching of fura-2 fluorescence due to the influx of extracellular Mn^{2+} induced by vasopressin. Verapamil as well as diltiazem inhibited vasopressin-stimulated Mn^{2+} influx in a dose-dependent manner up to 60% at concentrations of 200–400 μM. Furthermore, both inhibitors decreased significantly the frequency of phenylephrine-induced oscillation of $[Ca^{2+}]_i$. The experimental findings indicate that L-type channel blockers inhibit ROCCs in rat hepatocytes.

Verapamil; Diltiazem; Receptor-operated Ca^{2+} channel; Fura-2; Calcium oscillation; Rat hepatocyte

1. INTRODUCTION

An increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) was found to be caused by hormones, neurotransmitters, or membrane depolarisation. In excitable cells, voltage-dependent calcium channels (VDCCs) are responsible for the increase of $[Ca^{2+}]_i$ by the influx of extracellular Ca^{2+} across the plasma membrane [1]. In non-excitable cells, an increase of $[Ca^{2+}]_i$ results: (i) from the activation of intracellular Ca^{2+} stores, such as endoplasmatic reticulum, via the phospholipase C-linked inositol 1,4,5-trisphosphate cascade [2–4]; and (ii) from a receptor-mediated Ca^{2+} entry across the plasma membrane through receptor-operated Ca^{2+} channels (ROCCs) [5].

The quenching of fura-2 fluorescence by the vasopressin-stimulated influx of extracellular Mn^{2+} is an appropriate method for measuring flux rates through ROCCs [6]. Specific organic inhibitors for ROCCs are as yet not available, but it is well known that classical L-type VDCC blockers, like verapamil or diltiazem, influence liver metabolism, e.g. inhibit apolipoprotein B synthesis and secretion [7], inhibit bile acid synthesis [8], or inhibit c-*myc* expression in rat hepatocytes [9]. The reported effects of verapamil on Ca^{2+} entry in hepatocytes are controversial. Hughes et al. [10] found a completely inhibited vasopressin-induced Ca^{2+} influx by measuring the initial rate of $^{45}Ca^{2+}$ exchange, whereas Kass et al. [6] reported that verapamil did not affect the vasopressin-induced quenching of fura-2 fluorescence due to Mn^{2+} inflow. Therefore, we reinvestigated the influences of verapamil and diltiazem on agonist-stimulated Mn^{2+} inflow and studied their effects on the intracellular Ca^{2+} signal.

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Abbreviations: ROCCs, receptor-operated Ca^{2+} channels; $[Ca^{2+}]_i$, intracellular free calcium concentration; VDCCs, voltage-dependent Ca^{2+} channels.

2. MATERIALS AND METHODS

[Arg^8]-vasopressin, forskolin, verapamil and diltiazem were from Sigma (Deisenhofen, Germany). (−)-phenylephrine was from Serva (Heidelberg, Germany), fura-2/AM and collagenase H were from Boehringer (Mannheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

2.1. *Cell preparation and fura-2 loading*

Hepatocytes from male Wistar rats (150–250 g, 24 h fasted) were prepared by collagenase H perfusion of liver according to Berry and Friend [11]. For fura-2 loading and fluorescence measurement freshly isolated hepatocytes (> 85% intact cells with Trypan blue exclusion) were resuspended in Krebs-Henseleit medium saturated with 95% O_2/5% CO_2, containing (in mM): glucose (10), HEPES (10), lactate (2.1), pyruvate (0.3), β-hydroxybutyrate (0.6), acetoacetate (0.3), and $CaCl_2$ (1.3). The cell suspension (4 mg protein per ml) was incubated with 10 μM fura-2/AM for 20 min at 37°C in a shaking water bath and for additional 20 min at room temperature, thereafter centrifuged (300×*g*, 1 min) and resuspended in the same medium without fura-2/AM. The last step was repeated to wash out extracellular fura-2/AM. After this procedure, the intracellular fura-2 concentration was 60–80 μM as estimated from the fluorescence intensities (340 nm excitation) of the cell suspension. Cellular autofluorescence of unloaded hepatocytes accounted for < 15% of the total signal from fura-2 loaded hepatocytes. Loaded cells were stored at 0°C and used within 2 h.

2.2. *Measurement of Mn^{2+} influx*

Fura-2 loaded cells were transferred in a continuously stirred quartz cuvette maintained at 30°C in a Sigma ZWSII dual wavelength spectrophotometer. The fluorescence emission at the 340 nm and 360 nm

excitation wavelengths were recorded using a Schott KV500 cutoff filter. The initial fluorescence intensities of the hepatocyte suspension were arbitrary set to 1.0 at 340 nm and to 0.6 at 360 nm excitation for all cell preparations in order to get comparable results independently of different intracellular fura-2 concentrations.

2.3. *Measurement of* $[Ca^{2+}]_i$

1 ml of fura-2 loaded hepatocytes (0.4 mg protein per ml) was filled in a perifusion chamber mounted on the stage of an Olympus IMT-2 inverted fluorescence microscope and allowed to attach to the cover slip on the bottom of the chamber for 5 min at room temperature. Perifusion media with and without effectors were maintained at 30°C, saturated with 95% O_2/5% CO_2 and fed into the perifusion chamber by a peristaltic pump. The flow rate was 2 ml per min. The excitation wavelengths were 340 nm and 380 nm using appropriate interference filters in a Sutter Instruments filter wheel. The emission was set above 500 nm by a dichroic mirror. Fluorescence images were obtained from a Hamamatsu C2400–87 intensified charge-coupled device (CCD) camera. An IBM-compatible 386 computer digitized and stored the images. Ratio images (340 nm excitation/380 nm excitation) were calculated and analysed using the Olympus CUE/RMS software package.

3. RESULTS

The effect of verapamil on the vasopressin-induced fluorescence quenching due to the influx of extracellular Mn^{2+} is shown in Fig. 1. Whereas additions of verapamil and forskolin to fura-2 loaded hepatocytes did not affect the fluorescence at 340 nm excitation, indicating no change of $[Ca^{2+}]_i$, the addition of 500 μM $MnCl_2$ immediately decreased the fluorescence at both excitation wavelengths (360 nm not shown). According to Kass et al. [6], this decrease is caused by the binding of Mn^{2+} to traces of extracellular fura-2. The initial Mn^{2+}-dependent quenching was followed by a slow further decrease in fluorescence due to an unspecific Mn^{2+} influx into the cell or a slow efflux of fura-2. Subsequent addition of 10 nM vasopressin markedly enhanced the fluorescence quenching by a stimulated influx of Mn^{2+} into hepatocytes (Fig. 1A) [6]. This vasopressin-induced quenching was distinctly inhibited by 200 μM verapamil (Fig. 1B). The inhibitory effect of verapamil did not depend on the presence of forskolin and a similar degree of inhibition was found if manganese was added after vasopressin (Table I). The inhibition was dose-dependent up to 60% at concentrations of 200–400 μM (Fig. 2A). Similar results were obtained with diltiazem (Fig. 2B). The inhibition can be interpreted by a linear inhibition model as demonstrated by a linear dixon plot (data not shown). The apparent K_i for verapamil and diltiazem was 252 μM and 286 μM, respectively, from non-linear regression curves (Fig. 2).

In order to investigate whether verapamil or diltiazem really inhibit ROCCs we studied the influence of both inhibitors on intracellular calcium oscillations. Hepatocytes were stimulated with phenylephrine (< 5 μM), resulting in an oscillatory change of the ratio signal representing $[Ca^{2+}]_i$ (Fig. 3). The addition of 200 μM

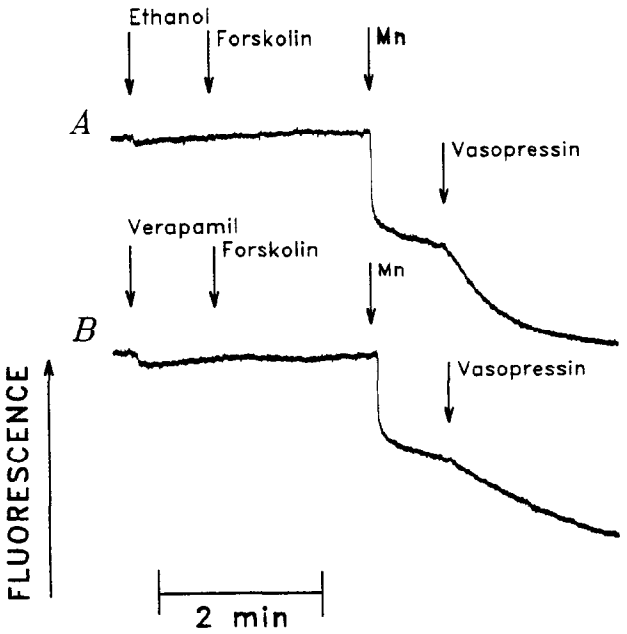

Fig. 1. Verapamil inhibited the vasopressin-induced fluorescence quenching of fura-2 loaded hepatocytes by the influx of extracellular Mn^{2+}. The figure shows the fluorescence intensity at 340 nm excitation. Additions were (A) 30 μl ethanol as control or (B) 200 μM verapamil dissolved in ethanol, 10 μM forskolin in order to produce a maximal response to vasopressin [6], 500 μM $MnCl_2$, and 10 nM vasopressin.

Table I

The influence of forskolin and of the sequence of additions on the effect of verapamil

	Mn^{2+} influx (arbitrary units/min)	
	Without forskolin	10 μM forskolin
Control (without verapamil)	17.6 ± 0.7	35.4 ± 5.5
Manganese before vasopressin	12.2 ± 3.2	16.2 ± 5.1
Manganese after vasopressin	9.9 ± 1.5	n.d.

Experiments were performed as in Fig. 1. The data are the mean ± S.E.M. from 3–5 experiments. n.d. = not determined.

Table II

The influence of verapamil or diltiazem on calcium oscillations

	Frequency of oscillation (spikes/10 min)	*n*
Without verapamil	3.9 ± 1.3	4
200 μM verapamil	1 1 ± 0.9 *	4
Without diltiazem	9.0 ± 1.8	5
200 μM diltiazem	3.5 ± 2.0 ***	5

Experiments were performed as in Fig. 3. The data are the mean ± S.E.M. from *n* experiments. * $P < 0.05$, *** $P < 0.001$ calculated from Student's paired *t*-test.

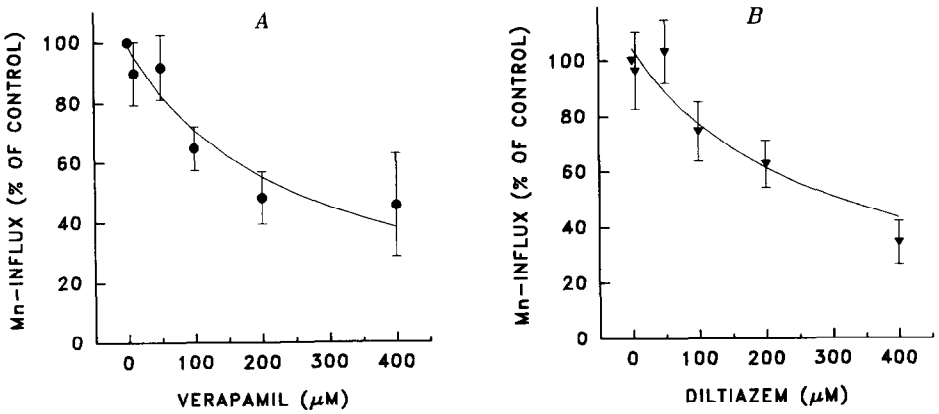

Fig. 2. The effect of verapamil or diltiazem on agonist-stimulated Mn^{2+} influx was dose-dependent. Experiments were performed as in Fig. 1 with various concentrations of verapamil (A) or diltiazem (B). The equation $y = V/(1+x/K_i)$ was used for non-linear regression resulting in apparent K_i = 252 μM or 286 μM for verapamil or diltiazem, respectively. Each point is the mean ± S.E.M. of at least 5 incubations.

verapamil or the same concentration of diltiazem distinctly decreased the frequency of Ca^{2+} oscillations without influencing the amplitude of oscillation (Fig. 3 and Table II).

4. DISCUSSION

This study demonstrates that verapamil as well as diltiazem are able to inhibit partly the vasopressin-stimulated Mn^{2+} influx at concentrations of 200–400 μM. For both inhibitors we estimated apparent K_i in the range of 200 μM. We conclude from these experiments that L-type channel blockers inhibit Ca^{2+} channels in the plasma membrane of hepatocytes which are possibly ROCCs. Our results are in agreement with the findings of Hughes et al. [10]. These authors found a 50% inhibition of the vasopressin-induced Ca^{2+} inflow by verapamil or nifedipine at concentrations of 50–100 μM by measuring the $^{45}Ca^{2+}$ exchange and the activity of glycogen phosphorylase a. In contrast to us, Kass et al. [6] reported that the rates of Mn^{2+} influx after addition of vasopressin alone or in combination with glucagon were not changed by verapamil up to 200 μM.

Glennon et al. [12] postulated in a recent paper that the increased agonist-induced quenching of hepatocytes loaded by the fura-2 ester was not an effect of the agonist on Mn^{2+} entry across the plasma membrane, but rather due to a penetration of Mn^{2+} into an intracellular organelle. This was concluded from a comparison to experiments with fura-2 injected cells where fura-2 was considered to be exclusively located in the cytosol. Three observations, in our opinion, are not in line with this interpretation and indicate that vasopressin-induced quenching results from Mn^{2+} inflow across the plasma membrane in fura-2/AM loaded cells, too. (i) Even Glennon et al. [12] found a stimulated Mn^{2+} entry

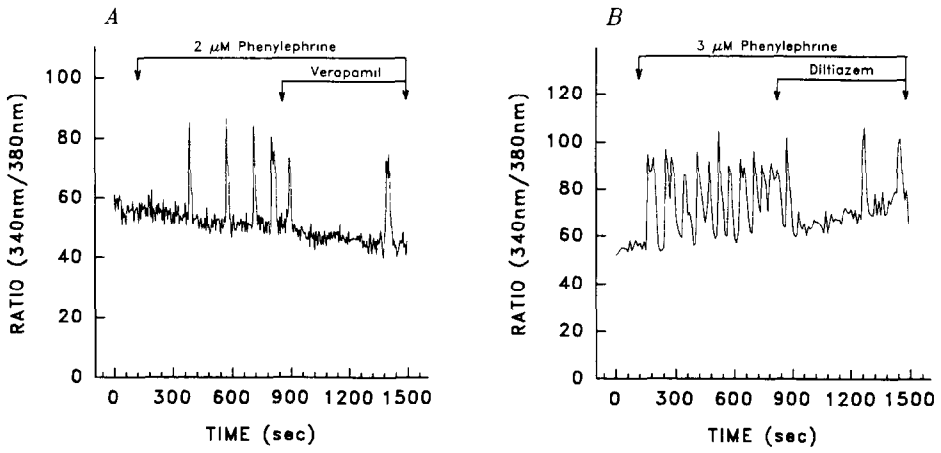

Fig. 3. Suppression of phenylephrine-induced oscillations of $[Ca^{2+}]_i$ by (A) 200 μM verapamil or (B) 200 μM diltiazem. The figure shows the ratio signal (340 nm excitation/380 nm excitation) of one characteristic cell of one experiment with verapamil or diltiazem, respectively. The effect of decreased frequency was observed in all oscillatory cells (cf. Table II).

into fura-2 injected hepatocytes by vasopressin indicating that the hormone activates channels in the plasma membrane. (ii) Comparable inhibitory effects on the influx rate of Mn^{2+} were observed if Mn^{2+} was added after vasopressin. In this case, an accumulation of Mn^{2+} prior to the addition of vasopressin, as argued by Glennon et al. [12], is excluded, so that the monitored quenching must result from the flux of Mn^{2+} across the plasma membrane. (iii) The effect of verapamil or diltiazem on the frequency of vasopressin-induced Ca^{2+} oscillations, as reported here, corresponds to the removal of extracellular Ca^{2+} from the medium [13]. After addition of the inhibitor, the first Ca^{2+} spike came in the normal time interval and thereafter the frequency of spikes was decreased. A possible explanation for this finding is that a hindered Ca^{2+} uptake into the cell caused by inhibited ROCCs delays the refilling of intracellular Ca^{2+} stores. Thus, a longer time interval is necessary for a new quantal Ca^{2+} release induced by inositol 1,4,5-trisphosphate [14]. To our knowledge, this study shows for the first time the influence of Ca^{2+} channel blockers on oscillation of $[Ca^{2+}]_i$ in non-excitable cells. Taken together with the effects on Mn^{2+} quenching and other effects reported in the literature [7–9] we conclude that the L-type channel blockers inhibit receptor-operated calcium channels in hepatocytes.

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